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(74) See (73)

(54) A method for production of new inhibitors of dipeptidyl peptidase IV

(55) Inhibitors; dipeptidyl peptidase IV; amino acid derivatives; heterocyclic amide structure; production; competitive inhibition; therapeutic; medicine, immunobiochemistry; pharmaceutical industry

(57) This invention relates to a method for production of new inhibitors of dipeptidyl peptidase IV based on the special amino acid derivatives with heterocyclic amide structure, that inhibit competitively the catalytic activity of the enzyme in the purified form and also in the normal or pathologically changed human and animal sera, in organs, tissues and cells of human, animal, plant, and microorganism origin, not only in vivo but also in vitro, and are used as potential therapeutics in metabolic processes under the regulatory control by dipeptidyl peptidase IV. The invention is important in use in medicine, particularly in immunobiology and pathology, and for the pharmaceutical industry.

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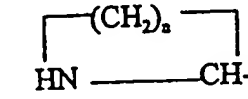
Claims:

1. A method for production of new inhibitors of dipeptidyl peptidase IV, **characterized** in that amino acid amide of the general formula I,

A-B (I)

is synthesized, wherein A and B are defined as follows:

A = α-aminocarboxylic acid of the structure H_2N-CHR-COOH (R= aliphatic, aromatic, or heterocyclic residue): for example, alanine, valine, leucine, serine, threonine, cysteine, methionine, proline, lysine, arginine, histidine, glutamic acid, glutamine, aspartic acid, asparagine, phenylalanine, tyrosine, tryptophan, norvaline, norleucine, ornithine, 2,4-diaminobutyric acid, α-aminobutyric acid, preferably isoleucine (always in the L-configuration), α-aminoisobutyric acid, in case of trifunctional amino acids also the corresponding N^{ω}- or C^{ω}- or O- or S-substituted derivatives in the L-configuration, preferably N^{ω}-acyl, C^{ω}- or O-benzyl amino acids, for example N^{ε}-4-nitrobenzyloxycarbonyl-L-lysine, O-benzyl-L-serine, O-benzyl-L-tyrosine, γ-benzyl ester of L-glutamic acid, β-benzyl ester of L-aspartic acid as well as the corresponding derivatives, particularly those ring-substituted with halogen, nitro-, hydroxy-, lower normal or branched alkyl- or alkoxy- residues, of L-phenylalanine, L-tyrosine, L-tryptophan, preferably 4-nitro-L-phenylalanine or α-iminocarboxylic acid of the structure



with n=2, 3, or 4, for example L-azetidine-2-carboxylic acid, L-proline, L-pipecolic acid, related compounds such as L-3,4-dehydroproline, L-thioproline as well as the corresponding derivatives substituted with halogen-, nitro-, hydroxy-, cyano-, lower normal or branched alkyl- or alkoxy residues, for example L-4-hydroxyproline.

B= special heterocyclic amines or heterocyclic amine-aldehydes: pyrroline, thiazoline, piperidine, morpholine, pyrazoline, pyrazolidine, piperazine, oxazoline, oxazolidine, imidazoline, imidazolidine, azetidine, aziridine, preferably pyrrolidine, thiazolidine, L-prolinal, L-thioprolinal, as well as the corresponding derivatives substituted with halogen-, nitro-, or alkyl residues, and their production is performed by starting from X-A-Y or X-A(Z)-Y (in case of trifunctional amino acids for A) by substitution with B, wherein A and B are defined as described above, X stands for an α-amino-protecting group commonly

used in peptide chemistry, preferably the t-butyloxycarbonyl residue, Z represents a common side chain-protecting group, preferably of the t-butyl-type (t-butyloxycarbonyl, t-butyl ester, O- or S-t-butyl) depending on the structure of the trifunctional amino acid, and Y means hydroxy, active ester, preferably pentafluorophenyl or N-hydroxysuccinimide ester, according to the method common in the peptide chemistry for attachment of the amide bond, desirably via the anhydride mixture technique or the active ester method, then the protecting groups used for X and Z are removed with the deblocking method common in the peptide chemistry for the above-mentioned protecting groups of the t-butyl type through acidolysis, and if necessary, the products are purified through re-crystallization or through column chromatography on Sephadex G10 or weakly acidic ion exchange resin.

2. A method for production of new inhibitors of dipeptidyl peptidase IV according to Claim 1, **characterized in** that the amino acid derivatives,

Ile-pyrrolidide
Ile-thiazolidide
Ile-prolinal
Ile-thioprolinal
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-pyrrolidide
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-thiazolidide
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-prolinal
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-thioprolinal,

represent the favorable compounds in respect to their inhibitory activities.

3. A method for production of new inhibitors of dipeptidyl peptidase IV according to Claim 1 and Claim 2, **characterized in** that these and/or their pharmaceutically acceptable salts inhibit the catalytic activities of enzymes in the pure form as well as in normal or pathologically changed human and animal organs, tissues, and cells of human, animal, plant, and microorganism origin not only in vivo but also in vitro and are important for the medicine as potential therapeutics in metabolic processes under the regulatory control by dipeptidyl peptidase IV.

Field of the Invention

The invention relates to the method for production of new inhibitors of dipeptidyl peptidase IV (DP IV) based on the special amino acid derivatives of a heterocyclic amide structure. The compounds of the invention inhibit competitively the catalytic activity of dipeptidyl peptidase IV and can be used as reversible DP IV-inhibitors in medical-biological processes in which the enzyme takes a functional part, and as potential diagnostics or therapeutics. The invention is suitable for application in medicine, veterinary medicine, pathobiochemistry, pharmacology, immunobiochemistry and for the pharmaceutical industry.

Prior Art

Dipeptidyl peptidase IV is an enzyme ubiquitously found in mammals. It is a serine protease with marked substrate specificity, that splits off consecutively dipeptides of the structure X_As-Pro and X_As-Ala from the N-terminal end of a peptide- or protein-chain, provided that there is no proline- or hydroxyproline residue in the third position of the sequence (see Küllertz et al., Dipeptidyl peptidase IV—Chemistry, biochemistry, and physiologic aspects. Contribution to active substance research, No. 11, Academy-Industry-Complex Research on Drugs 1981). Recent findings indicate that the dipeptidyl peptidase IV seems to be a physiologically and biochemically important enzyme that takes a functional part in a series of metabolic processes particularly in blood pressure regulation, blood coagulation, and proliferation processes (see G. Küllertz et al., Dipeptidyl peptidase IV—Biochemistry, physiology and pathobiochemistry. Contribution to active substance research, No.27, Academy-Industry-Complex Research on Drugs 1988). It is known that X_As-Pro- or X_As-Ala-dipeptide is effective as competitive inhibitors of the dipeptidyl peptidase IV, where their inhibitory activity is dependent on the structure of the N-terminal amino acid X_As. However, as a whole, their inhibitory activity does not seem very marked (H.U. Dermuth, Dissertation A., Math.-Nat. Faculty of Halle University 1981). Moreover, irreversible inhibitors (acyl enzyme inhibitors) of dipeptidyl peptidase IV of dipeptidyl-O-aroyl-hydroxylamine type have recently been reported (see H.U. Dermuth et al., J. Enzyme Inhibition (1988), 2, 129). About such inhibitors, toxicologic considerations can not be precluded in the in vivo studies. In addition, preference is

given to the reversible inhibition of the enzyme activity in case of therapeutic use of DP IV-inhibitors.

Object of the Invention

The object of the invention consists in making available very active inhibitors of the dipeptidyl peptidase IV that are easy to produce on the basis of special amino acid derivatives with heterocyclic amide structure, that inhibit competitively the catalytic activity of DP IV as very tolerable substances not only in vitro but also in vivo, where, though reserved for special use depending on the molecular structure, a gradual variation of their inhibitory potency can be achieved, and that could be important preferably in medicine not only in the field of diagnosis but also in the therapy.

Disclosure of the Invention

The invention is based on the subject to develop new inhibitors of the dipeptidyl peptidase IV of the amino acid amide type that inhibit reversibly the catalytic activity of DP IV and are distinguished themselves by the following advantages:

1. simple molecular structure
2. simple and therefore inexpensive production
3. targeted modulation of inhibitory potency through modification of the structure
4. favorable physical-chemical parameters in the sense of high penetrability
5. high bioavailability at the action site.

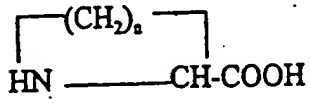
The subject is solved by synthesis of amino acid amides of the general formula 1

A-B (I)

wherein A and B are defined as follows:

A= α-aminocarboxylic acid of the structure H_2N-CHR-COOH (R= aliphatic, aromatic, or heterocyclic residue): for example, alanine, valine, leucine, serine, threonine, cysteine, methionine, proline, lysine, arginine, histidine, glutamic acid, glutamine, aspartic acid, asparagine, phenylalanine, tyrosine, tryptophan, norvaline, norleucine, ornithine, 2,4-diaminobutyric acid, α-aminoisobutyric acid, preferably isoleucine (always in the L-configuration), α-aminoisobutyric acid, in case of trifunctional amino acids also the corresponding N^{ω}- or C^{ω}- or O- or S-substituted derivatives in the L-configuration,

preferably N^{ω}-acyl, C^{ω}- or O-benzyl amino acids, for example N^{ε}-4-nitrobenzyloxycarbonyl-L-lysine, O-benzyl-L-serine, O-benzyl-L-tyrosine, γ-benzyl ester of L-glutamic acid, β-benzyl ester of L-aspartic acid as well as the corresponding derivatives, particularly those ring-substituted with halogen-, nitro-, hydroxy-, lower normal or branched alkyl- or alkoxy- residues, of L-phenylalanine, L-tyrosine, L-tryptophane, preferably 4-nitro-L-phenylalanine or α-iminocarboxylic acid of the structure



with n=2, 3, or 4, for example L-azetidine-2-carbonxylic acid, L-proline, L-pipecolic acid, related compounds such as L-3,4-dehydroproline, L-thioproline as well as the corresponding derivatives substituted with halogen-, nitro-, hydroxy-, cyano-, lower normal or branched alkyl- or alkoxy residues, for example L-4-hydroxyproline.
B= special heterocyclic amines or heterocyclic aminoaldehydes: pyrroline, thiazoline, piperidine, morpholine, pyrazoline, pyrazolidine, piperazine, oxazoline, oxazolidine, imidazoline, imidazolidine, azetidine, aziridine, preferably pyrrolidine, thiazolidine, L-prolinal, L-thioprolinal, as well as the corresponding derivatives substituted with halogen-, nitro-, or alkyl residues.

The following amino acid derivatives are some preferable compounds as DP IV-inhibitors according to the invention:

Ile-pyrrolidide
Ile-thiazolidide
Ile-prolinal
Ile-thioprolinal
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-pyrrolidide
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-thiazolidide
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-prolinal
N^{ε}-4-nitrobenzyloxycarbonyl-Lys-thioprolinal

Production of the amino acid amides of the invention as reversible inhibitors of DP IV is performed by starting from X-A-Y or X-A(Z)-Y (in case of trifunctional amino acids for A) by substitution with B, wherein A and B are defined as described above, X stands for an α-amino protective group usually used in the peptide film (see E. Wünsch,

Synthesis of peptides in Houben Weyl Volume 15/1, Methods of organic chemistry, Ed. E. Müller, Georg-Thieme-Verlag Stuttgart 1974), preferably a t-butyloxycarbonyl residue, Z represents a common side chain-protecting group, favorably of t-butyl-type, depending on the nature of the trifunctional amino acid, namely t-butyloxycarbonyl residue is used for the protection of N^{ω}-amino function and t-butyl ester for blocking of ω-fixed carboxy group of t-butyl ester, and t-butyl residue for hydroxy- or thiol functions, and Y means hydroxy, active ester, preferably pentafluorophenyl- or N-hydroxysuccinimide ester, according to the usual method in the peptide chemistry for attachment of the amide bond, namely N,N-dicyclohexylcarbodiimide, N,N-dicyclohexylcarbodiimide/additives (preferably 1-hydroxybenzotriazol), activated ester, anhydride mixture, acid chloride (see E. Wünsch, above-mentioned), or 2-(1H-benzotriazol-1-yl-)-1,1,3,3-tetramethyluronium salt (see R. Knorr et al., Abstracts 20th Europ. Peptide Symposium Tübingen 1988) or benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium salt (see A. Fournier et al., Int. J. Peptide Protein Res., 1988, 31, 86) for the protected amino acid amides of the general formulas II and III.

X-A-B (II)

X-A(Z)-B (III)

with A, B, X, and Z in the above-mentioned definition.

The attachment of the amide bond between A and B is preferably performed via the anhydride mixture technique or the active ester method, where N-hydroxysuccinimide- or pentafluorophenyl ester are favorably used. The protected amino acid amides of the general formulas II and III that have been obtained can be purified, if necessary, through re-crystallization or through column chromatography on silica gel or LH-20. After simultaneous or successive removal of the protecting groups for X and Z with the deblocking method common in the peptide chemistry (see E. Wünsch, see above) for the above-mentioned preferred protecting groups, t-butyloxycarbonyl, t-butyl ester, and t-butyl, through acidolysis (particularly with HCl/acetic acid; HCl/acetic acid ester, HCl/dioxane; trifluoroacetic acid, if necessary, in the presence of a cation-scavenger), the desired amino acid amides of the general formula I are obtained which, if necessary, can be purified through re-crystallization or through column chromatography on Sephadex G10 or weakly acidic ion exchange resin.

Amino acid derivatives with heterocyclic amide structure of formula I obtained according to the invention and/or their pharmaceutically acceptable salts can inhibit, as reversible inhibitors of dipeptidyl peptidase IV, the catalytic activity of the enzyme in the purified form and also in normal or pathologically changed human and animal sera, in organs, tissues and cells of human, animal, plant and microorganism origin, not only in vivo but also in vitro, and be used as potential therapeutics in the metabolic processes under the regulatory control by dipeptidyl peptidase IV, favorably in blood pressure regulation, blood coagulation, cell proliferation, and also in processing of biologically active proline-containing peptide.

The invention will be explained based on the Examples but the Examples do not limit the invention at all.

Examples

Following abbreviations are used:

Amino acid symbols in accordance with the IUPAC-IUB Joint Commission on Biochemical Nomenclature, Biochem. J., 219, 345 (1984).

Spro	L-thioproline (L-thiazolidine-4-carboxylic acid)
AcOH	acetic acid
Boc	t-butyloxylcarbonyl
CAIBE	chlorocarboxylic acid isobutyl ester
DC	thin layer chromatogram, -chromatographic
DP IV	dipeptidyl peptidase IV
d.Th.	of the theory
EE	acetic acid ethyl ester
EtOH	ethanol
Fp	melting point
h	hour(s)
i.Vak	in vacuum
LM	solvent
MeOH	methanol
Min.	minutes

NEM	N-ethylmorpholine
OPfp	pentafluorophenyl ester
pNA	4-nitroanilide
RT	room temperature
SC	column chromatography, -chromatographic
TEA	triethylamine
THF	tetrahydrofuran
$Z(NO_2)$	4-nitrobenzyloxycarbonyl

"The usual reprocessing" means as follows:

After terminated coupling reaction, each raw product is dissolved in acetic acid ester and the solution is washed successively twice with water (saturated with NaCl), three times with 5% $KHSO_4$-solution, twice with water, three times with saturated sodium hydrogen carbonate solution, and three times with water. The organic phase is dried over Na_2SO_4 and the raw product is isolated by evaporation of the solvent in vacuum.

The following solvent systems (in volume) were used for the thin layer chromatography on silica gel precoat plate (Silufol UV254, GSSH):

BAE	benzene-acetone-acetic acid	70+30+1.5
BAW	2-butanol-formic acid-water	75+15+20
BEWE	1-butanol-acetic acid-water-acetic acid ester	20+20+20+20
BPEW	1-butanol-pyridine-acetic acid-water	30+20+6+24
CM	chloroform-methanol	90+10
EPEW	acetic acid ester-pyridine-acetic acid-water	90+15+4.5+2.3

For determination of the inhibitory activity of the DP IV-inhibitors synthesized according to the invention, the Ki-values were determined from the intercept of at least 3 lines obtained by plotting according to Dixon (in: J. Lasch, Enzyme kinetics, Fischer-VLG. Jena 1987), 1/vi against [I].

vi – measured initial velocity of DP IV-catalyzed hydrolysis of the substrate Ala-Pro-pNA.

[I] – Concentration of amino acid amide studied as a DP IV-inhibitor in the formulation for measurement

The measurement was performed at pH6.3 in 0.04 M phosphate buffer. The ionic

strength was 0.125, caused by potassium chloride. The temperature of the formulation for measurement was 30°C. Determination of the value vi was performed 3 times for each concentration of the substrate and the inhibitor.

Example I

N'-4-nitrobenzyloxycarbonyl-L-lysine pyrrolidide · hydrochloride (H-Lys[Z(NO_2)]-N ⊂ · HCl)

A 2.95 g portion of Boc-Lys[Z(NO_2)]-OH was dissolved in 30 ml THF and treated with 880 µl of NEM and 900 µl of CAIBE at -15°C. After 6 Min. 573 µl of pyrrolidine at -15°C was added. The mixture was stirred at -15°C for 1 h and at room temperature overnight. The re-processing was performed as usual. The amorphous Boc-Lys[Z(NO_2)]-N ⊂ obtained after drying i.Vak. was dissolved in 20 ml of 1.1N HCl/AcOH and kept still at RT for 30 Min. The product was subjected to precipitation with ether and then re-crystallized from MeOH/ether. Further purification is performed by SC on Sephadex G10 with 0.1M AcOH as the elution solvent.

Yield:

Fp: 157 - 160°C

$[\alpha]_D^{20}$: +9.67° (c=1, AcOH)

DC: homogenous in BAW, BEWE, and BPEW

Ki: $(3.46 \pm 0.5) \times 10^{-7}$ M

Example II

L-valine-pyrrolidide · hydrochloride (H-Val-N ⊂ · HCl)

A 1.086 g portion of Boc-Val-OH was dissolved in 20 ml of EE and treated with 640 µl of NEM and 650 µl of CAIBE at -20°C. After 8 Min. 41.3 µl of pyrrolidine was added and the mixture was stirred at -20°C for 1 h and at room temperature overnight. The re-processing was performed as usual. The oily Boc-Val-N ⊂ was treated with 3N HCl/EE at room temperature for 30 Min. After concentration of the LM i.Vak., the product was crystallized from EtOH/EE as colorless needles.

Yield: 620 mg (60.3% d.Th.)

Fp: 178-180°C

$[\alpha]_D^{20}$: +33.93° (c = 1, AcOH)

DC: homogenous in BAW, BEWE, and BPEW

Ki: $(4.75 \pm 0.7) \times 10^{-7}$ M

Example III

L-isoleucine-pyrrolidide · hydrochloride (H-Ile-N  · HCl)

A 1.98 g portion of Boc-Ile-OPfp was dissolved in 15 ml of THF and treated with 450 µl of pyrrolidine and 280 µl of TEA at 0°C. The mixture was stirred at 0°C for 1 h and at RT for 1 hr. After removal of LM i.Vak. the residue was dissolved in EE and washed with H_2O, $KHSO_4$-solution and H_2O, and dried over Na_2SO_4. EE was removed i.Vak. and the oily Boc-Ile-N was treated at RT with 15 ml of 1.1 N HCl/AcOH for 30 Min. The product was precipitated first with ether, evacuated, and dried in the desiccator over KOH and P_2O_5, and then re-crystallized from isopropanol/diisopropylether.

Yield: 760 mg (68.8% d.Th.)

Fp: 179 - 184°C

$[\alpha]_D^{20}$: +29.31° (c=1, AcOH)

DC: homogenous in BAW, BEWE, and BPEW

Ki: $(2.43 \pm 0.1) \times 10^{-7}$ M

Example IV

L-thioproline-pyrrolidide · hydrochloride

IV.1. Boc-SPro-N

A 888 mg portion of Boc-SPro-OH was dissolved in 10 ml of THF and treated with 484 µl of NEM and 495 µl of CAIBE with stirring after cooling to -20°C. After 8 Min. 443 µl of pyrrolidine was added at -20°C and the reaction mixture was stirred at -20°C for further 1 h and at room temperature overnight. After concentration i. Vak., the residue was dissolved in EE and re-processed as usual. After dissolution of the oily residue in n-hexane, crystallization started.

Yield: 416 mg (38% d.Th.)

Fp: 108 - 109°C

$[\alpha]_D^{26}$: -154.2° (c=0.62, AcOH)

DC: homogenous in BAE, CM, and EPEW

IV.2. H-SPro-N ⟨⟩ · HCl

A 265 mg portion of Boc-Spro-N ⟨⟩ was dissolved in 3 ml of 1.1N HCl/AcOH, treated with 300 µl of thioanisol, and kept still at RT for 30 Min. Then concentration i.Vak. was performed, and the residue was treated with ether and re-crystallized from $CHCl_3$/ether.

Yield: 182 mg (88% d.Th.)

Fp: 164-166°C

$[\alpha]^{20}_D$: -122.7° (c=0.62, AcOH)

DC: homogenous in BAW, BEWE, and BPEW

Ki: $(3.95 \pm 0.4) \times 10^{-5}$ M

Example V

L-isoleucine-thiazolidide · hydrochloride

V.1. Boc-Ile-N ⟨S⟩

A 2.31 g portion of Boc-Ile-OH was dissolved in 10 ml of THF and treated with 1.27 ml of NEM and 1.3 ml of CAIBE with stirring at -20°C. After 8 Min. 1.26 g of thiazolidine-hydrochloride and additional 1.27 ml NEM were added at -20°C, and the reaction mixture was stirred at -20°C for 1 h and at room temperature overnight. After concentration of the mixture i. Vak., the residue was dissolved in EE and re-processed as usual. The oily residue was purified by flash-chromatography on silica gel with ether/n-hexane (1:1).

Yield: 952 mg (31% d.Th.)

$[\alpha]_D^{26}$ (oil): -14.1° (c=0.6, AcOH)

DC: homogenous in BAE, CM, and EPEW

V.2. H-Ile-N⌐S¬ · HCl

A 790 mg portion of Boc-Ile-thiazolidide was dissolved in 8 ml of 1.1N HCl/AcOH, treated with 800 µl of thioanisol, and kept still at RT for 30 Min. Then the mixture was concentrated i.Vak., and the product was precipitated with ether.

Yield: 584 mg (94% d.Th.)

Fp: 116 - 120°C

$[\alpha]_D^{26}$: +18.6° (c=0.77, AcOH)

DC: homogenous in BPFW, BEWE, and BAW

Ki: $(1.23 \pm 0.2) \times 10^{-7}$ M